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FILING DATE: *March 17, 2004*
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Bala Walid Mohamed	RATHINASAHAPATHI FOUAD	Gainesville, Florida Gainesville, FL 32608

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

INCREASED STRESS TOLERANCE AND ENHANCED YIELD IN PLANTS

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☑ Specification *Number of Pages* 16 ☐ CD(s), Number

☑ Drawing(s) *Number of Sheets* 6 ☐ Other (specify)

☐ Application Date Sheet. See 37 CFR 1.76

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[Page 1 of 2]

Respectfully submitted,

SIGNATURE

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Date March 17, 2004

REGISTRATION NO. 43,218 *(if appropriate)*

Docket Number: 10457-055

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☑ Applicant claims small entity status. See 37 CFR 1.27

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Complete if Known	
Application Number	
Filing Date	Concurrently Herewith
First Named Inventor	Bala RATHINASABAPATHI
Examiner Name	
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1003	530	2003	265	Plant filing fee	
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1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
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FEE CALCULATION (continued)

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1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

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SUBMITTED BY				(Complete *(if applicable)*)	
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Signature				*Date*	03/17/2004

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TITLE OF THE INVENTION

INCREASED STRESS TOLERANCE AND ENHANCED YIELD IN PLANTS

Government Support

This invention was made through support from the USDA-Agricultural Research Service, Grant No. NRICGP 2001-35318-10947. The government has certain rights in this invention.

BACKGROUND

The non-protein amino acid β-alanine is a precursor of pantothenate (vitamin B5) in all plantS; and the osmoprotectant β-alanine betaine in most members of Plumbaginaceae (Hanson et. al., 1991). β-Alanine betaine is a product of three sequential methylations of β-alanine (Rathinasabapathi et. al., 2001). While beta-alanine itself can be an osmoprotectant, in certain plants it is methylated to a more effective osmoprotectant called beta-alanine betaine.

Bacteria make beta-alanine by decarboxylating aspartic acid. In *Escherichia coli*, this reaction is catalyzed by the product of panD gene encoding L-aspartate-α-decarboxylase. Aspartate decarboxylation reaction is not known in plants. Plants appear to use a variety of other ways to synthesize beta-alanine. Thus, engineering strategies to increase β-alanine pool in plants has potential applications for improving nutritional quality and abiotic stress tolerance of crops.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods and compositions for increasing stress tolerance in plants.

It is another object of the present invention to provide plants and plant cells which have increased stress resistance.

It is a further object of the present invention is to increase biomass yield in plants. Preferably, enhanced biomass yield is accomplished by increasing leaf carbon dioxide concentration. Preferably still, enhanced growth and biomass is achieved by transforming plants with a polynucleotide molecule that decarboxylates aspartic acid.

Another object of the present invention is to enhance production of pantothenate in select plants.

The objects of the present invention, and others, may be accomplished with a method of increasing stress resistance in a plant, comprising expressing an aspartate decarboxylase in the plant. More preferred, an embodiment of the invention pertains to transforming plants with the panD gene of *E. coli*.

The objects of the present invention may also be accomplished with a method of increasing stress resistance in a plant cell, comprising expressing an aspartate decarboxylase in the plant cell.

The objects of the present invention may also be accomplished with a plant or a plant cell transformed with a nucleic acid, which encodes an aspartate decarboxylase.

Thus, the present invention also provides a method of producing such a plant or plant cell, by transforming a plant or plant cell with the nucleic acid which encodes the aspartate decarboxylase.

The present invention also provides an isolated and purified aspartate decarboxylase having the amino acid sequence of SEQ ID NO: 2.

The present invention also provides a method of producing the aspartate decarboxylase described above, comprising culturing host cells which have been transformed with a nucleic acid encoding the aspartate decarboxylase under conditions in which the aspartate decarboxylase is expressed, and isolating the aspartate decarboxylase.

In another embodiment, the present invention provides an isolated and purified enzyme having aspartate decarboxylase activity, wherein the amino acid sequence of the enzyme has a homology of from 70% to less than 100% to SEQ ID NO: 2.

The present invention also provides a method of producing the enzyme described above, comprising culturing host cells, which have been transformed with a nucleic acid encoding the enzyme under conditions in which the enzyme is expressed, and isolating the enzyme.

Brief Description of the Drawings

Figure 1. Diagram showing the generation of β-alanine through the α-decarboxylation of L-aspartic acid by the bacterial L-aspartate-α-decarboxylase (ADC).

Figure 2. ADC self-processing and assembly. Auto-cleavage between Gly24 and Ser25 amino acid residue generates pyruvoyl active group at the α-subunit. Three α-subunits and three β-subunits assemble together with one unprocessed protein to form the active ADC.

Figure 3. PCR screening for eight pMON-panD putative transgenic plants and two pMON-R5 putative transgenic plants. Two positive control TOPO-panD vector and one negative control no DNA.

Figure 4. Southern blot analysis for transgenic tobacco lines. 10 μg genomic DNA was digested with Hind III and hybridized with 32P-panD probe. Seven transgenic plants had a single gene insertion arrows indicate lines with single gene insertion pattern.

Figure 5. Tobacco transgenic lines with different levels of *AAC(3)-III* and *panD* transcription. The lower panel shows ethidium bromide stained gel with equal load (20μg total RNA each lane)

Figure 6. ADC induction. *E. coli* strain BL21-DE3 harboring pETB-panD vector was induced for 3 h with different IPTG concentrations showing over-expression of recombinant ADC at 16.8 KDa.

Figure 7. ADC purification. Left, 10-20 % Tris-tricine gel for the DEAE-Sepharose collected fractions. Right, Western blot for the DEAE-sepharose fractions using anti-His-CooH antibody. The antibody recognized the unprocessed π-peptide (16.8 KDa) and the α-subunit (14 KDa) of the ADC.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described herein, transformation and expression of aspartate decarboxylase in plant cells and plants produces increased biomass yield. Such plants are also expected to be also more resistant to drought, heat stress, salt stress and freezing stress.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1982) and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989); Methods in Plant Molecular Biology, Maliga et al, Eds., Cold Spring

Harbor Laboratory Press, New York (1995); Arabidopsis, Meyerowitz et al, Eds., Cold Spring Harbor Laboratory Press, New York (1994) and the various references cited therein.

The term "plant" includes whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. Preferred plants include rice, corn, wheat, cotton, peanut, and soybean. Thus, in one embodiment of the present invention, the stress tolerance of a plant can be enhanced or increased by increasing the amount of protein available in the plant, preferably by the enhancement of the aspartate decarboxylase gene in the plant.

Thus, one embodiment of the present invention are plant cells carrying a polynucleotide that encodes an aspartate decarboxylase, and preferably transgenic plants carrying such polynucleotide.

As used herein, the term "enhancement" means increasing the intracellular activity of one or more enzymes in a plant cell and/or plant which are encoded by the corresponding DNA. Enhancement can be achieved with the aid of various manipulations of the bacterial cell. In order to achieve enhancement, particularly over-expression, the number of copies of the corresponding gene can be increased, a strong promoter can be used, or the promoter- and regulation region or the ribosome binding site which is situated upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same manner. In addition, it is possible to increase expression by employing inducible promoters. A gene can also be used which encodes a corresponding enzyme with a high activity. Expression can also be improved by measures for extending the life of the mRNA. Furthermore, enzyme activity as a whole is increased by preventing the degradation of the enzyme. Moreover, these measures can optionally be combined in any desired manner. These and other methods for altering gene activity in a plant are known as described, for example, in Methods in

Plant Molecular Biology, Maliga et al, Eds., Cold Spring Harbor Laboratory Press, New York (1995).

A gene can also be used which encodes a corresponding or variant enzyme with a high activity. Preferably the corresponding enzyme has a greater activity than the native form of the enzyme, more preferably at least in the range of 5, 10, 25% or 50% more activity, most preferably more than twice the activity of the native enzyme.

In the context of the present application, a polynucleotide sequence is "homologous" with the sequence according to the invention if at least 70%, preferably at least 80%, most preferably at least 90% of its base composition and base sequence corresponds to the sequence according to the invention. According to the invention, a "homologous protein" is to be understood to comprise proteins which contain an amino acid sequence at least 70% of which, preferably at least 80% of which, most preferably at least 90% of which, corresponds to the amino acid sequence disclosed in (Gish and States, 1993); wherein corresponds is to be understood to mean that the corresponding amino acids are either identical or are mutually homologous amino acids. The expression "homologous amino acids" denotes those which have corresponding properties, particularly with regard to their charge, hydrophobic character, steric properties, etc. Thus, the protein may be from 70% up to less than 100% homologous to SEQ ID NO: 2.

Homology, sequence similarity or sequence identity of nucleotide or amino acid sequences may be determined conventionally by using known software or computer programs such as the BestFit or Gap pairwise comparison programs (GCG Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wis. 53711). BestFit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of identity or similarity between two sequences. Gap performs global alignments: all of one sequence with all of another similar sequence using the method of Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970). When using a sequence alignment program such as BestFit, to determine the degree of sequence homology, similarity or identity, the default setting may be used, or

an appropriate scoring matrix may be selected to optimize identity, similarity or homology scores. Similarly, when using a program such as BestFit to determine sequence identity, similarity or homology between two different amino acid sequences, the default settings may be used, or an appropriate scoring matrix, such as blosum45 or blosum80, may be selected to optimize identity, similarity or homology scores.

The present invention also relates to plant cells or plants transformed with polynucleotides which contain the complete gene with the polynucleotide sequence corresponding to the *E. coli* panD gene (Merkel and Nichols, 1996) or fragments thereof, and which can be obtained by screening by means of the hybridization of a corresponding gene bank with a probe which contains the sequence of said polynucleotide molecule or a fragment thereof, and isolation of the DNA sequence.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate those cDNAs or genes which exhibit a high degree of similarity to the sequence of the panD gene.

Polynucleotide sequences according to the invention are also suitable as primers for polymerase chain reaction (PCR) for the production of DNA which encodes an enzyme having aspartate decarboxylase activity.

Oligonucleotides such as these, which serve as probes or primers, can contain more than 30, preferably up to 30, more preferably up to 20, most preferably at least 15 successive nucleotides. Oligonucleotides with a length of at least 40 or 50 nucleotides are also suitable.

The term "isolated" means separated from its natural environment.

The term "polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, and can denote an unmodified RNA or DNA or a modified RNA or DNA.

The term "polypeptides" is to be understood to mean peptides or proteins which contain two or more amino acids which are bound via peptide bonds.

The polypeptides for use in accord with the teachings herein include polypeptides corresponding to *E. coli* aspartate decarboxylase, and also includes those, at least 70% of which, preferably at least 80% of which, are homologous with the polypeptide corresponding to *E. coli* aspartate decarboxylase, and most preferably those which exhibit a homology of least 90% to 95% with the polypeptide corresponding to *E. coli* aspartate decarboxylase and which have aspartate decarboxylase activity. Thus, the polypeptides may have a homology of from 70% to up to 100% with respect to *E. coli* aspartate decarboxylase.

The invention also relates to transforming plant cells and plants with polynucleotide sequences which result from *E. coli* panD gene by degeneration of the genetic code. In the same manner, the invention further relates to DNA sequences which hybridize with *E. coli* panD gene or with parts of *E. coli* panD gene. Moreover, one skilled in the art is also aware of conservative amino acid replacements such as the replacement of glycine by alanine or of aspartic acid by glutamic acid in proteins as "sense mutations" which do not result in any fundamental change in the activity of the protein, i.e. which are functionally neutral. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair the function thereof, and may even stabilize the function.

In the same manner, the present invention also relates to employing DNA sequences which hybridize with *E. coli* panD gene or with parts of *E. coli* panD gene. Finally, the present invention relates to DNA sequences which are produced by polymerase chain reaction (PCR) using oligonucleotide primers which result from *E. coli* panD gene. Oligonucleotides of this type typically have a length of at least 15 nucleotides.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a polynucleotide will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing).

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° C., and a wash in 1× to 2×SSC (20×SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.5× to 1×SSC at 55 to 60° C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65° C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA—DNA hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984): Tm=81.5oC.+16.6 (log M)+0.41 (% GC)−0.61 (% form)−500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary

target sequence hybridizes to a perfectly matched probe. Tm is reduced by about 1° C. for each 1% of mismatching; thus, Tm, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with approximately 90% identity are sought, the Tm can be decreased 10° C. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4° C. lower than the thermal melting point (Tm); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10° C. lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20° C. lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45° C. (aqueous solution) or 32° C. (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (2000).

Thus, with the foregoing information, the skilled artisan can identify and isolate polynucleotides which are substantially similar to the present polynucleotides utilized in accord with the teachings herein. In so isolating such a polynucleotide, the polynucleotide can be used as the present polynucleotide in, for example, increasing the abiotic stress and/or biomass yield of a plant.

One embodiment of the present invention is methods of screening for polynucleotides which have substantial homology to the polynucleotides of the present invention, preferably those polynucleotides encode a protein having aspartate decarboxylase activity.

The polynucleotide sequences of the present invention can be carried on one or more suitable plasmid vectors, as known in the art for plants or the like.

In one embodiment, it may be advantageous for propagating the polynucleotide to carry it in a bacterial or fungal strain with the appropriate vector suitable for the cell type. Common methods of propagating polynucleotides and producing proteins in these cell types are known in the art and are described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1982) and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989).

In another preferred embodiment the polynucleotide comprises *E. coli* panD gene, polynucleotides which are complementary to *E. coli* panD gene, polynucleotides which are at least 70%, 80% and 90% identical to *E. coli* panD gene; or those sequence which hybridize under stringent conditions to *E. coli* panD gene, the stringent conditions comprise washing in 5×SSC at a temperature from 50 to 68° C. Thus, the polynucleotide may be from 70% up to less than 100% identical to E. coli panD gene.

In another preferred embodiment the polynucleotides of the present invention are in a vector and/or a host cell. Preferably, the polynucleotides are in a plant cell or transgenic plant. Preferably, the plant is selected from the group consisting of wheat, corn, peanut cotton, oat, tobacco, and soybean plant. In a preferred embodiment, the polynucleotides are operably linked to a promoter, preferably an inducible promoter designed for expression in plants.

In another preferred embodiment, the present invention provides a method for making aspartate decarboxylase protein, comprising culturing the host cell carrying the polynucleotides of the invention for a time and under conditions suitable for expression of aspartate decarboxylase, and collecting the aspartate decarboxylase protein.

In another preferred embodiment, the present invention provides a method of making a transgenic plant comprising introducing the polynucleotides of the invention into the plant.

In another preferred embodiment, the present invention provides method of increasing the abiotic stress tolerance of a plant in need thereof, comprising introducing the polynucleotides of the invention into said plant.

In another preferred embodiment, the present invention provides method of increasing the biomass yield of a plant, comprising introducing the polynucleotides of the invention into said plant under conditions where said polynucleotides are expressed.

Methods, vectors, and compositions for transforming plants and plant cells in accordance with the invention are well-known to those skilled in the art, and are not particularly limited. For a descriptive example see Karimi et al., TRENDS in Plant Science, Vol. 7, No. 5, May 2002, pp. 193-195, incorporated herein by reference.

Aspartate α-decarboxylation

Prokaryotes have a unique route of β-alanine synthesis through the α-decarboxylation of L-aspartic acid (fig. 1). The enzyme catalyzing this reaction, L-aspartate-α-decarboxylase (ADC) was identified (Cronan, 1980), and the *Escherichia coli panD* gene for ADC was cloned, over-expressed, and the enzyme purified and characterized (Ramjee et. al., 1997, Albert et. al., 1998 and Chopra et. al., 2002). Based on 14C-aspartate radio labeling experiments, this enzyme was not detected in plants (Naylor et. al., 1958); and no sequence homologues are known in eukaryotes (Rathinasabapathi et. al., 2000).

ADC self-processing

Bacterial aspartate decarboxylase is an unusual enzyme. It translates as an inactive precursor protein which goes through self-processing to give α and β subunits (102 and 24 amino acid residues, respectively; figure 2). Three of each of these subunits

and one unprocessed π-peptide are assembled to form the active ADC enzyme (Ramjee et. al., 1997). As an unusual prokaryotic enzyme that have no homologues in eukaryotes, an important question needs to be answered; when the bacterial ADC protein is expressed in transgenic eukaryotic systems, will it behave the same way as in prokaryotes? Will it self-process and get assembled properly to give an active enzyme? To answer these questions, the idea of expressing ADC in transgenic plants seemed to be tempting. The best tool to study the ADC at the protein level in transgenic eukaryotic system is western blot; thus, over-expressing ADC in an efficient microbial expression system could facilitate ADC purification to be used for developing its specific polyclonal antibodies.

Cloning the *panD* gene from *E. coli*

A 429 bp fragment containing the aspartate decarboxylase open reading frame (ORF) was amplified using specific primers from *E. coli* DH5α genomic DNA as a template. The PCR product was cloned using TOPO-TA cloning kit *panD* DNA sequence was confirmed by sequencing. The pUC-panD vector complemented an *E. coli* mutant (strain AB543) defective in β-alanine biosynthesis, which confirmed that the cloned gene is coding for an active aspartate decarboxylase enzyme. This clone was used for further sub-cloning into plant expression vectors.

Tobacco leaf disks transformation

E. coli panD gene was sub-cloned under *35S* promoter in pMON-R5 plant expression vector, which has the *AAC (3)-III* gene that gives the transgenic plant resistance for kanamycin. The generated vector was named pMON-D-A9. The tobacco leaf-disks were maintained under kanamycin selection starting from the third day after the co-cultivation with Agrobacterium and were sub-cultured every 2-3 weeks. No kanamycin-resistant shoots were obtained with the control treatments. A total of 10 and 29 independent putative transgenic plants were generated from the leaf disks infected with Agrobacterium harboring pMON-R5 and pMON-D-A9 vectors respectively. All these plants were maintained under kanamycin till the rooting stage and then moved to the soil.

Genomic DNA from putative *panD* and pMON-R5 transgenic tobacco plants were isolated for PCR screening with *panD* specific primers. All the *panD* putative transgenic plants were positive and amplified the expected 429 bp PCR product confirming the *panD* insertion, while there was no PCR product with two pMON-R5 transgenic plants (fig. 3). To know the number of insertions in each transgenic plant, Southern analysis was conducted. Seven transgenic plants out of the thirteen tested showed a pattern consistent with a single gene insertion (fig 4). Kanamycin-resistant phenotype segregation in F2 of transgenic lines confirmed the single gene insertion.

Total RNA from 22 *panD* and 4 pMON-R5 putative transgenic plants were analyzed in RNA blots, probed with 32P-labeled DNA of either *AAC (3) III* (kanamycin resistance gene) or *panD*. As expected all the samples were positive for *AAC (3) III* expression. Some of the transgenics had detectable levels of *panD* expression. The expression levels for the two genes were variable between lines (fig 5).

ADC purification using pET Blue-2 expression system

The *panD* gene was amplified with introducing BspH I site on the ATG start codon at the 5' end and Pvu II site at the 3' end primers. The PCR product was digested with the two restriction enzymes and cloned directly into Nco I, Pvu II digested pET Blue-2 vector. The *panD* sequence was placed between the ribosomal binding site right before the *panD* ATG without any extra amino acids residues and a fusion peptide at the 3' end that had a His-tag. The resulting vector was named pETB-panD and sequenced. The vector was introduced to BL21-DE3 strain for protein induction with IPTG.

Figure 6 representing SDS-PAGE for DE3:pETB-panD induction with different IPTG concentrations for 3 h. The level of ADC expressed at molecular weight of 16.8 KDa is positively correlated to the IPTG concentration. The absence of lower band at 14 KDa suggests that the protein was not processed under the conditions of the experiment. The induced protein was also recognized with anti-His-COOH specific monoclonal antibody.

For protein purification, DE3:pETB-panD strain was grown in LB media and when reached 0.8 OD600 the culture was induced with 100 μM IPTG for 3 hours. The harvested cells were extracted with Bug Buster Protein extraction Reagent (Novagen) and the soluble protein was loaded onto 5 ml Ni++ column (Pro Bond purification system, Invitrogen) and eluted with 250 mM Imidazole. The eluted protein was loaded onto 2 ml of DEAE-Sepharose column. The proteins were eluted with liner gradient (0-1M KCl), protein fractions separated on SDS-PAGE and blotted proteins were subject to western analysis (fig. 7) using Anti-His-CooH monoclonal antibody (Novagen). The fractions have the recombinant ADC (F-4 and F-5) was assayed for enzyme activities and used for developing ADC native and SDS-denatured polyclonal antibodies.

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The teachings of the references cited throughout the specification are incorporated herein in their entirety by this reference to the extent they are not inconsistent with the teachings herein. It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

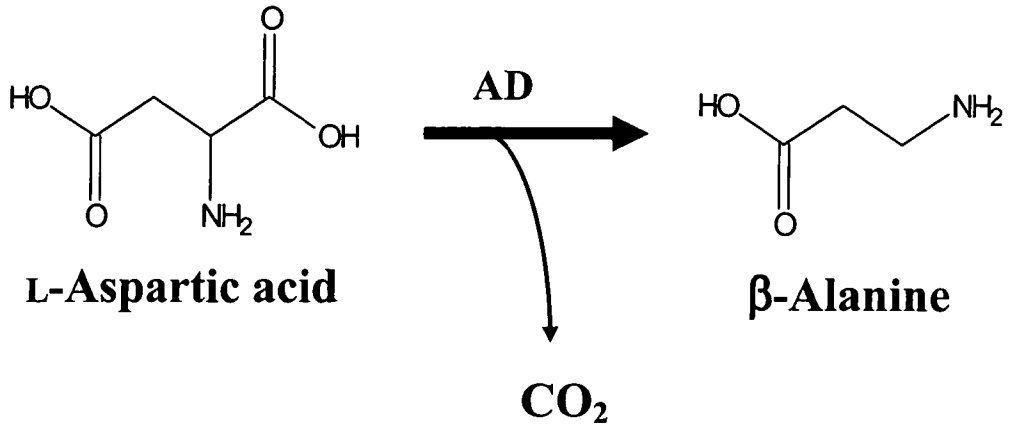

FIG. 1

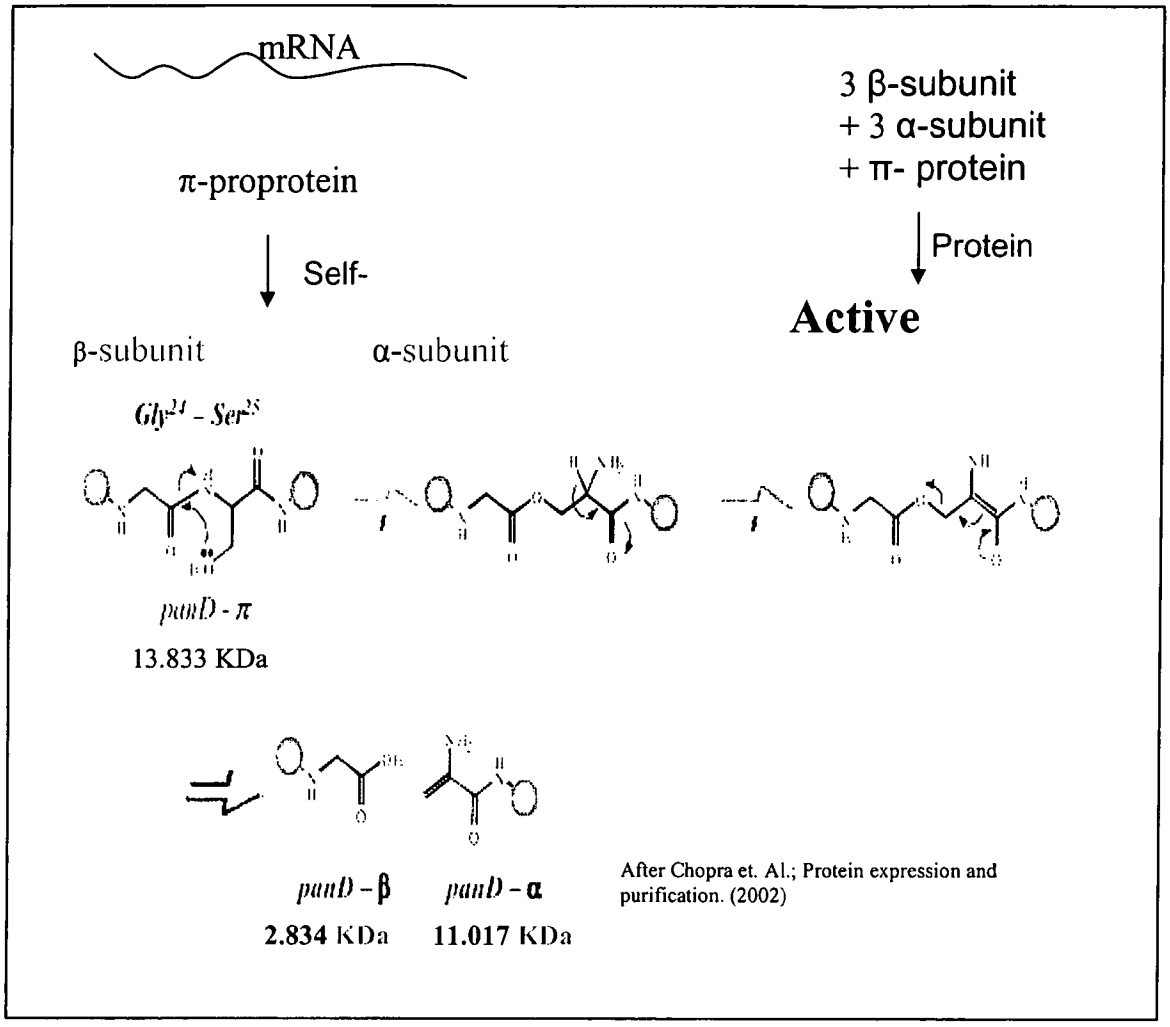
mRNA
π-proprotein
Self-
β-subunit
α-subunit
Gly24 - Ser25
panD - π
13.833 KDa
panD – β
2.834 KDa
panD – α
11.017 KDa
3 β-subunit
+ 3 α-subunit
+ π- protein
Protein
Active
After Chopra et. Al.; Protein expression and purification. (2002)

FIG. 2

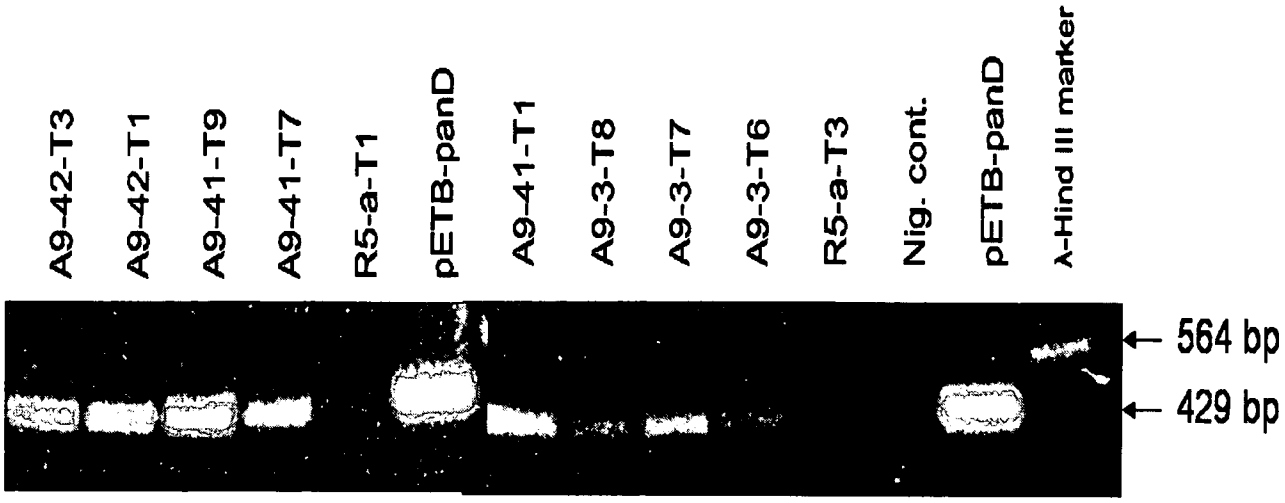
A9-42-T3
A9-42-T1
A9-41-T9
A9-41-T7
R5-a-T1
pETB-panD
A9-41-T1
A9-3-T8
A9-3-T7
A9-3-T6
R5-a-T3
Nig. cont.
pETB-panD
λ-Hind III marker
564 bp
429 bp

FIG 3

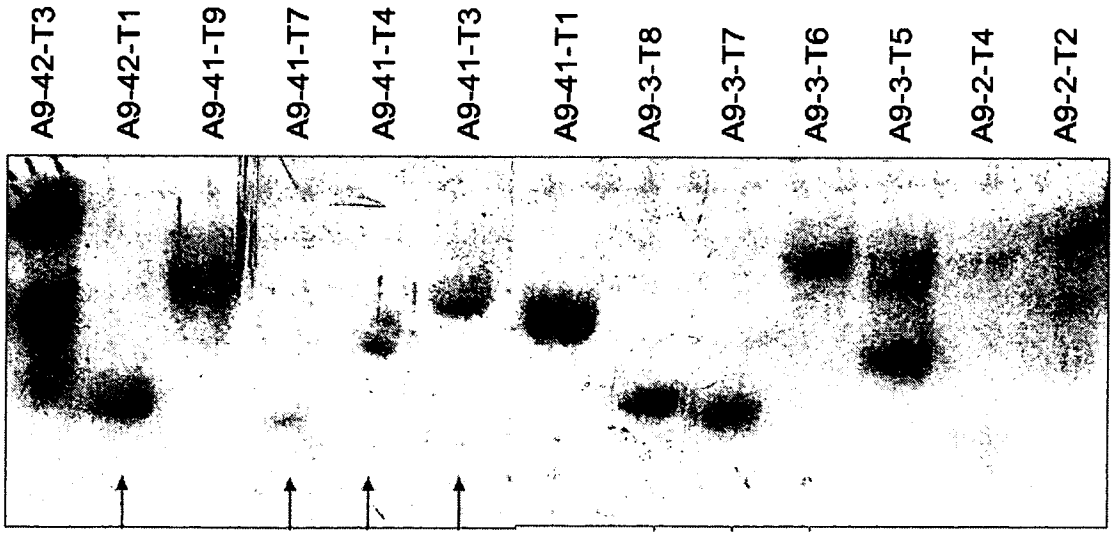
A9-42-T3
A9-42-T1
A9-41-T9
A9-41-T7
A9-41-T4
A9-41-T3
A9-41-T1
A9-3-T8
A9-3-T7
A9-3-T6
A9-3-T5
A9-2-T4
A9-2-T2

FIG. 4

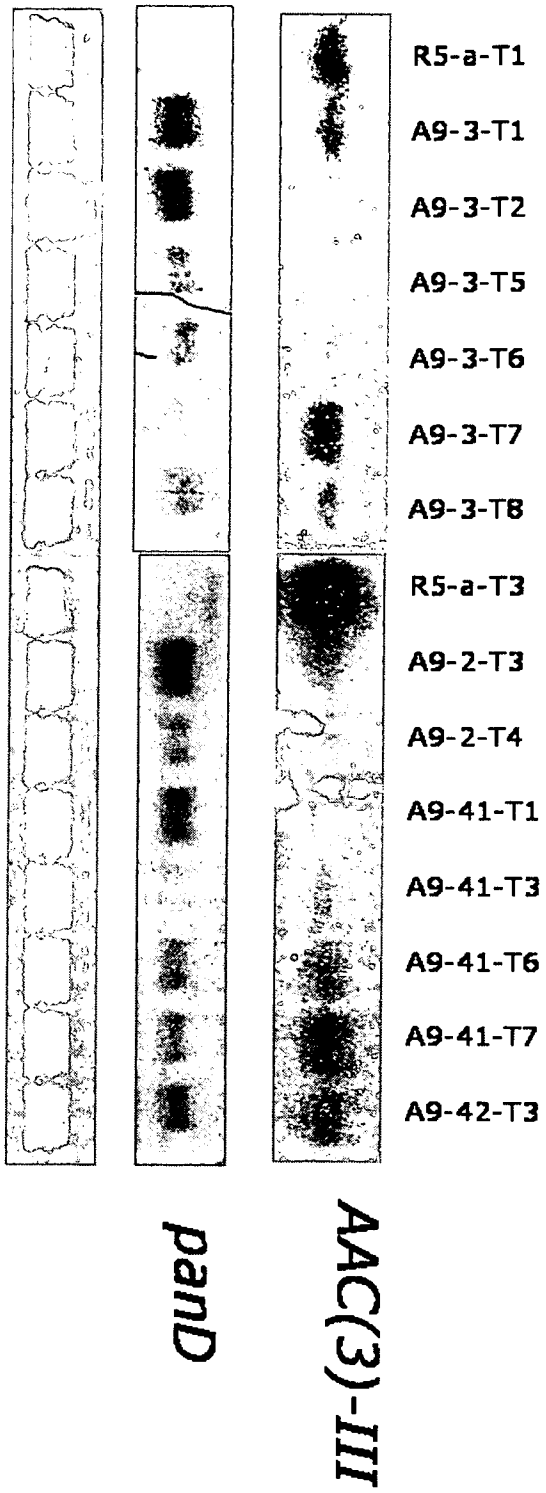
R5-a-T1
A9-3-T1
A9-3-T2
A9-3-T5
A9-3-T6
A9-3-T7
A9-3-T8
R5-a-T3
A9-2-T3
A9-2-T4
A9-41-T1
A9-41-T3
A9-41-T6
A9-41-T7
A9-42-T3
panD
AAC(3)-III

FIG. 5

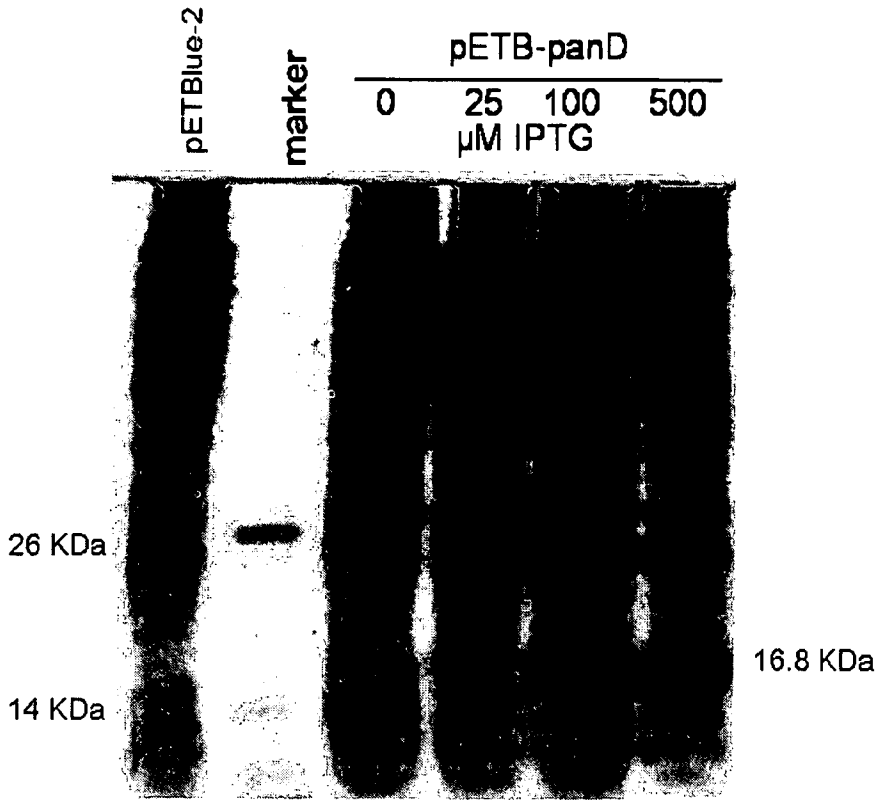
pETBlue-2
marker
pETB-panD
0
25
100
500
μM IPTG
26 KDa
14 KDa
16.8 KDa

FIG. 6

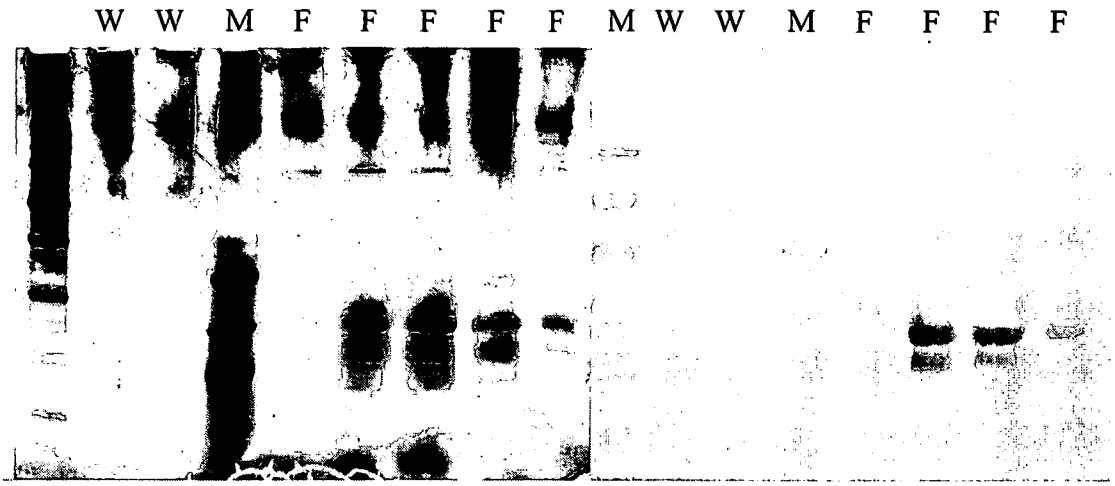

FIG. 7